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(54) Title: LIPOSOMES

(57) Abstract

Liposomes having covalently bound PEG moieties on the external surface have improved serum half-life following intravenous administration.

LIPOSOMES

The present invention relates to liposomes bearing polyethylene glycol (PEG) moieties covalently linked to the external surface.

Many ways have been sought to prolong the half life of liposomes in the circulation. Methods have included incorporation of gangliosides in the lipid bilayer, as described by Allen, T.M. et al. Biochim. Biophys. Acta 818: 205-210, and coating the liposomal surface with molecules such as glycosides, as described by Gosh, P. and Bachawat, B.K. Biochim. Biophys. Acta. 632: 562-572, and poloxamers, as described by Senior J. CRC Critical Reviews in Therapeutic Drug Carriers 3: 123-193 (1987).

There is however, a need for a technique which increases the surface hydrophilicity of liposomes (whether these are small unilamellar vesicles or multilamellar vesicles or large unilamellar vesicles of defined size) while quantitatively retaining aqueous solutes, without crosslinking the vesicles and without conferring on the vesicle a net charge.

A particular problem arises in the use of liposomes to modify the circulation lifetime characteristics of magnetic resonance imaging agents such as Gd-DTPA described by Unger et al., Radiology, 171 81-85 (1989) and Tilcock et al., Radiology, 171: 77-80 (1989). For use as a perfusion agent it would be desirable to increase the circulation lifetimme of liposomal Gd-DTPA.

Once administered i.v., the liposomes are subject to numerous interactions with plasma proteins (eg. HDL) and the Reticulo-endothelial system (RES) which result in destabilisation and clearance of the vesicles from the circulation. Methods that have been employed to date to improve vesicle stability in the circulation have been to

dioeylphosphatidyl choline and dioleylphosphatidyl ethanolamine and most pref rably the liposomes contain aqueous Gd-DTPA.

The invention further provides a process comprising treating liposomes with a reactive derivative of polyethylene glycol, preferably 2,2,2-trifluoroethanesulphonyl (tresyl) monomethoxy PEG. Tresyl monomethoxy PEG (TMPEG) and its production is described in our co-pending British application no. 8824591.5.

Preferably the reaction between the reactive PEG derivative and the liposomes is conducted in aqueous solution at ambient or physiological temperatures. The reaction occurs at near neutral pH, for instance in physiological buffer but is faster and more extensive at pH9-10. By controlling the ratio of reactive PEG derivative to liposomes, the number of PEG moieties linked to the liposomes may be controlled.

Poly(ethylene glycol) (PEG) is a linear, water-soluble polymer of ethylene oxide repeating units with two terminal hydroxyl groups:

$$HO(CH_2CH_2O)_nCH_2CH_2OH$$

PEG's are classified by their molecular weights, thus PEG 6000, for example, has a molecular weight of about 6000 and n is approximately 135.

PEG's can be covalently linked to proteins by a variety of chemical methods. We have used tresyl chloride (2,2,2-trifluoroethane sulphonyl chloride) to activate the single free hydroxyl group of monomethoxy PEG 5000 (MPEG) but other tresyl halides and other reactive derivatives of MPEG may be used. By having the other hydroxyl group of PEG "blocked" as the unreactive methyl ether, the possibility of producing PEG activated at both ends, which would give rise to cross-linked lipids in the coupling stage, is avoided.

The phospholipids phosphatidylethanolamine (PE) and

liposomes of the present invention is in the delivery of MR imaging agents such as Gd:diethylenetriaminepentacedic acid chelate.

The invention further provides the use of liposomes having PEG moieties bound to their external surfaces in therapeutic and diagnostic methods practised on the human or animal body, for instance as delivery means for drugs and for contrast agents for Magnetic resonance (MR) imaging. The invention provides a therapeutic or diagnostic process comprising intravenous administration of an effective, non toxic amount of a PEG-bearing liposomes as hereinbefore described containing a diagnostic or therapeutic agent to a human or non-human animal in need thereof.

The invention will now be illustrated by the figures of the accompanying drawings which:

Fig. 1. shows a comparison of the clearance of PEGylated SUVs and unPEGylated SUVs from the circulation in mice.

Fig. 1A: SUVs of composition DSPC:PE:Cholesterol (molar ratio 0.4:0.1.5) either PEGylated ( ) or untreated ( ) were injected iv into mice (0.4mg/25g mouse). Blood levels of CF (dose ± se, 5 animals) are shown; ^{3}H phospholipid clearance was similar (not shown).

Fig. 1B: and 1C: Identical conditions to Fig. 1A except that the SUV preparation had been centrifuged to 100,000g for 1 hr to remove larger vesicles and the injected dose was 0.8mg/25g mouse. Both CF Fig. 1B clearance and ^{3}H phospholipid clearance Fig. 1c are shown for PEGylated () and unPEGylated () vesicles.

The invention will now be illustrated by the following Examples:

NaCl containing 0.05M sodium phosphate buffer, pH 7.5 (PBS) at 10 mg total lipid/ml. 0.1ml samples of vesicles were incubated with solutions of TMPEG prepared in PBS (final concentrations 0-170 mg/ml) for 2 hours at room temperature. Samples were partitioned by adding samples (0.05 ml) to a biphasic system (1 ml of top phase and 1 ml of bottom phase of a phase system of 5% (w/w) PEG 6000 and 5% (w/w) Dextran T500 in 0.15M Nacl containing 0.01M sodium phosphate, pH 6.8, mixing the systems and measuring the radioactivity in samples taken from the mixture immediately after mixing (total) and from the top and bottom phases after phase separation was completed (20 min).

The results in Table 1 show that exposure of the liposome to TMPEG increases their partition into the PEG-rich top phase. This indicates that PEG has become attached to the liposome, presumably by the covalent attachment to the amino group of the EPE.

TABLE 1

The effect of TMPEG on the partitioning behaviour of multilamellar vesicles of EPE/EPC (2:8).

FINAL TMPEG	PARTITION (%)			n
(mg/ml)	Top Phase	Interface	Bottom Phase	
0.0	9.1±4.7	84.5± 4.1	6.4± 2.4	9
2.0	14.5±5.4	80.2± 4.2	5.3± 1.6	3
8.0	44.9±6.3	50.8± 6.5	4.3± 0.4	3
12.5	74.7±9.5	20.1±10.5	5.2± 1.4	3
25.0	96.3±7.8	3.1± 3.6	4.6± 0.8	4
50.0	89.3	6.5	4.5	1
100.0	88.8	5.1	6.1	1
170.00	89.3	6.5	4.2	1

Naphosphate buffer, pH 8.5). To measure liposomal retention of water-soluble molecules during the coupling reaction and subsequent procedures, Carboxyfluorescein was partially purified and entrapped at 0.15M as described by Senior et al., Biochim. Biophys. Acta 839: 1-8 (1985). 0.5ml SUV were incubated with an equal volume of TMPEG, prepared in PBS (0.125M NaCl buffered with 0.05M Naphosphate buffer, pH 8.5) at 125 mg/ml. for 2 hours at room temperature (Ratio of TMPEG to total DPPE is 6.25). The vesicles were then separated from unreacted TMPEG by gel filtration on Sepharose 4B-CL and partitioned as in Example 1 in a phase system of 5% PEG 8000 (Union Carbide) and 5% Dextran T500 (Pharmacia) in 0.15M NaCl containing 0.01M sodium phosphate, pH 6.8. The results in Table 3 show that exposure of the liposomes to TMPEG increases their partition into the PEG-rich top phase compared with vesicles treated only with buffer (control). This suggests that PEF has been covalently linked to the amino group of the DPPE. PEGylation proceded without the loss of the entrapped CF.

Table 3

Phase Partitioning of PEGylated and unPEGylated SUVs.

VESICLES	PARTITION[1] (%)		
Phase	Top Phase	Interface	Bottom
Untreated	1.4 ± 0.2	36.0 ± 5.0	62.5 ± 5.1
TMPEG-treated	96.5 ± 1.0	1.4 ± 1.1	2.1 ± 0.4

[1] mean ± n = 6

20 ul samples were removed and partitioned in a phase system of 1.5ml top phase and 1.5ml bottom phase of a 5% PEG 8000 (Union Carbide) and 5% Dextran T500 (Pharmacia) system prepared 0.15M NaCl buffered with sodium phosphate pH 6.8 at room temperature. Samples of top and bottom phase were removed for counting 20 min after the phase had been mixed and allowed to separate. This phase system was selected so that the partitioning of the untreated vesicles into the top phase was extremely low (>5%); the majority of the vesicles were approximately equally divided between the bottom phase and the bulk interface.

EXAMPLE 5

The time course and pH dependency of the PEGylation reaction using a two-fold excess of TMPEG to the DOPE present at the outer surface of LUVettes are used in Example 4. At pH 8-9 incubation with TMPEG rapidly caused a time dependant transfer of vesicles to the top phase. At pH 7.5 the reaction was considerably slower and at pH 7.0 there was virtually no transfer to the top phase. In a separate experiment in which the bottom phase and interface partitioning was also measured it is seen that at pH 7.2, although top phase partitioning does not alter there was decrease in bottom phase partitioning with an increase in interface partitioning, indicating that PEGylation proceed at pH 7.2 albeit more slowly than at higher pHs. At pH 8 the partitioning moves from the bottom phase to the interface and then to the top phase; at pH 9 and 10 vesicles are moved rapidly from the interface and bottom phase to the top phase. Thus the PEGylation reaction is very sensitive to pH and appropriate choice of conditions of time and pH can determine the degree of PEGylation. The extent of PEGylation can also be controlled by the amount of TMPEG used. Treating 100nm Luvettes of DOPE/DOPC (0.2:0.8) at pH 9.0 with varying molar ratios of TMPEG increased partitioning into the top phase

Measurement of the fraction f amino groups (from PE) exposed at the outer surface of the LUVettes, made by the method of Hope, M.J. and Cullis. P.R. J. Biol. Chem. 262: 4360-4366 (1987) in 0.05M TNBS in borate buffer at pH 8.5, gave values of 47% for DOPC:DOPE vesicles (8:2), close to the theoretical value of 50% for equal distribution of the PE between the inner and outer surfaces. PEGylation caused a decrease in the PE content detectable by this assay, suggesting covalent attachment of the MPEG to the free NH_2 group of PE. For example, when a 3-fold mole excess of TMPEG to outer PE was added to DOPC:DOPE vesicles of 7:3 molar ratio for 1 hour, the percentage of outer PE PEGylated was 36%; when a 6-fold molar excess was added, this percentage PEGylation increased to 45%.

20% top phase, 60% interface and 20% bottom phase. Treatment with serum caused an immediate (within 1 min) alteration in the vesicle surface properties indicated by their partit ion: 0% top phase, 40% interface and 60% bottom phase. The plasma proteins alone partitioned mainly to the bottom phase (68% bottom, 32% top; Partition coefficient $=0.47 \pm 0.02$, n=4). Thus it appears that the SUVs are immediately coated with serum proteins which then cause the vesicles to partition with similar characteristics to the proteins. PEGylation of the SUVs increased their partition into the top phase (almost 100%); on exposure to serum there was a change in their partition towards the interface and the bottom phase, but importantly this process was very slow compared with the virtually instantaneous effect of serum on unPEGylated SUVs. Since the partitioning behaviour relates to the sum of the forces imposed by the PEGylation and serum binding, and with the former is not a linear function, it is not simple to determine whether the effect of serum on partition is equal for the PEGylated and for the unPEGylated liposomes. This could, however, be determined with a detailed dose response analysis of the effect of PEGylation on the partition coefficient so that the influence of serum could be determined at various parts of the dose response curve in "PEG-equivalents". This would establish whether serum had different effects on the PEGylated and unPEGylated liposomes. The order of magnitude differences in partition behaviour suggests that PEGylation slows down the adsorption of serum components onto the vesicles.

Separation of the SUVs exposed to serum by gel chromatography gave vesicles which showed partitioning behaviour close to that of the vesicles before exposure. Thus the interaction between vesicles and serum is reversed by reisolation of the vesicles.

These experiments also demonstrate that the altered

Table 5

Stabilisation of 100nm LUV tte latency to serum (2hr, 37°) by PEGylation

DOPC:DOPE molar ratio	Latency (%)	
	UnPEGYLATED	PEGylated
8:2	35	35
7:3	55	83
6:4	90	95
5:5	92	99

EXAMPLE 9

PEGylation does not alter the relaxivity of encapsulated Gd-DTPA

Gd-DTPA was encapsulated in LUVettes composed of DOPC:DOPE 7:3 by the method of Tilcock et al Radiology 171: 77-80 (1989).

Half of the sample was PEGylated with TMPEG (molar ratio of TMPEG: PE on outer surface of 3:1). Both control and PEGylated samples were diluted in saline buffer (139 mM NaCl, 10 m Hepes, 6mM KCl, pH 8.5) to give four samples with effective Gd concentrations of 2, 1, 0.5, and 0.25 mM (calculated as described by Tilcock et al., Radiology 171: 77-80 (1989) given the known trap volume of the vesicles, the lipid concentration and assuming the concentration of entrapped Gd-DTPA was 0.67M.) Samples of 10-12 ml were imaged with a Toshiba 0.5T MRT-50A whole body scanner. Relaxivites are obtained from linear regressions of 1/T1 (spin lattice relaxation time constant) against the effective Gd-DTPA concentration. These were unaffected by PEGylation of the vesicles.

EXAMPLE 10

PEGylation of SUVs decreases their in vivo clearance

SUVs of composition DSPC:PE:Cholesterol (molar ratio 0.4:0.1:0.4) (0.2ml containing 0.4mg phosphpholipid) were

CLAIMS

1. Liposomes having covalently bound PEG moieties on the external surface.

2. Liposomes according to claim 1 wherein the lipid bilayers consist of a mixture of dioleylphosphatidylcholine (DOPC) and dioleylphosphatidylethanolamine (DOPE).

3. Liposomes according to claim 2 wherein the lipid bilayers consist of a 7:3 to 5:5 molar ratio of DOPC to DOPE.

4. Liposomes according to any one of claims 1 to 3 comprising an aqueous phase containing Gd:diethylenetriaminepentaacetic acid (Gd-DTPA).

5. A pharmaceutical composition comprising an aqueous suspension of liposomes according to any one of claims 1 to 4 and a pharmaceutically acceptable carrier or diluent.

6. Liposomes according to any one of claims 1 to 4 for use in a method for the treatment of the human or animal body or in a diagnostic method practised on the human or animal body.

7. Use of liposomes according to any one of claims 1 to 4 in the preparation of a medicament for use in a method of treatment of the human or animal body or a method of diagnosis practised on the human or animal body.

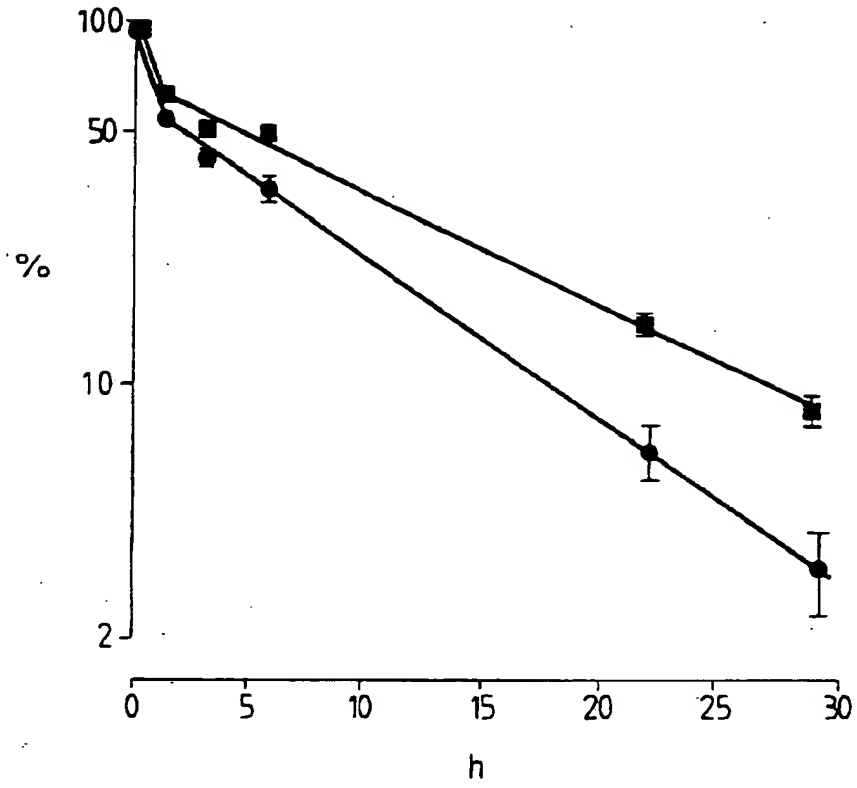
8. Use according to claim 8 of liposomes comprising an aqueous phase containing a contrast agent in a diagnostic method comprising magnetic resonance imaging of the human or animal body.

9. A process for producing a liposome according to any one of claims 1 to 4 comprising treating liposomes with a reactive derivative of polyethylene glycol.

10. A process according claim 9 wherein the reactive derivative is 2,2,2-trifluoroethane sulphonyl-monomethoxy-polyethylene glycol.

11. A therapeutic or diagnostic method comprising

Fig. 1A.



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/01262

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 9/127, A 61 K 49/00, A 61 K 47/48

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0171946 (TECHNICON INSTR. CORP.) 19 February 1986 see page 4, lines 13-25; page 7, lines 30-35; page 8, lines 22-31; page 9, lines 25-34; claims	1
Y	--	1-10
Y	GB, A, 2185397 (COSMAS-DAMIAN LTD) 22 July 1987 see page 1, lines 37-42; page 6, lines 41-53	1-10
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Y	STN File Server, (Karlsruhe), Chemical Abstracts, volume 109, no. 3, 1988, (Columbus, Ohio, US), C. Hofmann et al.: "Transfer of functional insulin receptors to receptor-deficient target cells", see abstract no. 17441m, & Endocrinology (Baltimore), 122(6), 2865-72	1-10
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* Special categories of cited documents: [12]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th February 1990	27.03.90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8901262

SA 31851

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 13/03/90
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